Indian J. Phys. **65B** (6) 587-594 (1991)

Spectroscopic characteristics and biological activity against ehrlich ascites carcinoma cells of some phenanthrenic alkaloids

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Abstract: The absorbance, fluorescence and phosphorescence characteristics of aristolochic acid, aristololactam, N-methyl aristololactam and aristololactam-β-D-glucoside have been evaluated from their UV-visible spectra, determination of their emission and excitation spectra, quantum yield and the life time of their excited states. Biological activities of these compounds towards Ehrlich Ascites Carcinoma cells in Swiss Albino mice have been determined by (i) the trypan blue test, (ii) loss of transplantability, (iii) morphological changes and (iv) the incorporation of radiolabelled precursor into DNA, RNA and protein synthesis. Comparative studies show that the cytotoxic property is in the order aristolochic acid (AA) aristololactam-β-D-glucoside (ADG) $>$ aristololactam (AL) $>$ N-methyl aristololactam (ALM).

Keywords: Aristolochic acid, aristololactam, aristololactam-β-D-glucoside, N-methyl aristololactam, Ehrlich ascites carcinoma cells, tumour cell-drug interaction.

PACS No: 87.15. Mi

1. Introduction

Aristolochia group of alkaloids and their glucoside derivatives have attracted recent attention for their antimicrobial, antitumour and various other biological properties (Kupchan and Doskotsch 1962, Kamatsh *et al* 1973, Chen and Zhu 1987). Some of the compounds of this group have been successively tested for the therapy of tuberculosis, chronic bronchitis, pneumococcal diseases and in the treatment of cancer (Chen and Zhu 1987, Cassady *et al* 1990). Recently, we have shown that one of its derivatives aristololactam-β-D-glucoside interacts with DNA by a mechanism of intercalation (Chakraborty *et al* 1989a), however, the antitumour

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properties of some of the derivatives of Aristolochia group including aristololactam, *N*-methyl aristololactam and aristololactam-β-D-glucoside (Figure 1) have not yet

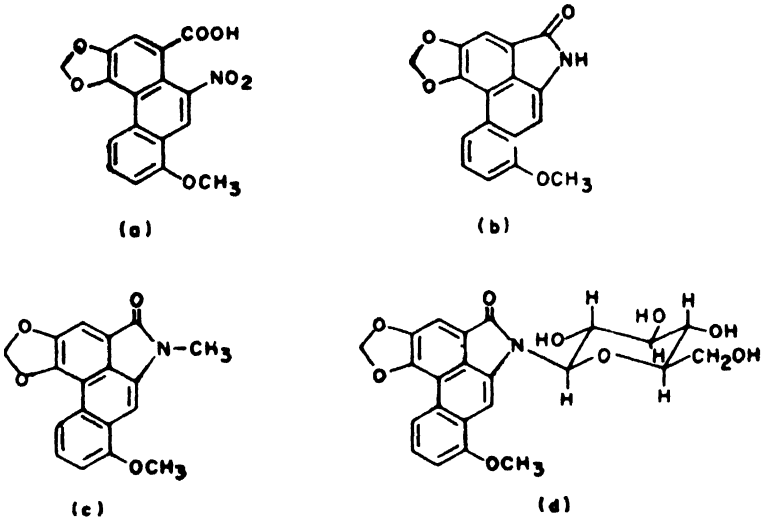

Figure 1. Chemical structure of (a) AA, (b) AL, (c) ALM and (d) ADG.

been reported so far. Present study deals with some spectroscopic properties and biological activities of these compounds towards Ehrlich Ascites Carcinoma cells on a comparative methods.

2. Materials and methods

The phenanthrenic group of alkaloids used in this study were isolated and characterized (Chakraborty *et al* 1989b). Dimethyl sulphoxide (DMSO), toluene, 1, 4-bis (5-phenyl oxazolyl) (POPOP), 2,5-diphenyl oxazole (PPO) were obtained from Sigma Chemical Co., St. Louis, M.O., U.S.A. [^{3}H] thymidine, [^{3}H] uridine and [^{3}H] leucine were obtained from Bhabha Atomic Research Centre, Bombay, RPMI 1640 medium with glutamine (without $NaHCO_3$) were obtained from Gibco BRL, New York. The medium was dissolved in water containing 3.7 g/lit $NaHCO_3$. All analytic grade chemicals and deionized double distilled water were used throughout.

Ehrlich Ascites Carcinoma (EAC) cells were obtained by the courtsey of Chittaranjan National Cancer Research Centre, Calcutta and were maintained by weekly intraperitoneal transplantation in Swiss Albino mice (male) weighing 18-22 g available from our Animal House Laboratory. EAC cells were harvested 6-8 days after inoculation by intraperitoneal puncture. The cells and accompanying fluid were mixed with two volumes of deionized water to lyse any red cell present and the mixture was centrifuged at 2000 rpm for 3 mins. The supernatant fluid was decanted and discarded, and the packed tumour cells were resuspended in 0.145 M NaCl solution. Then the cells were further diluted to the desired cell count as measured by haemocytometer.

Absorbance, fluorescence and phosphorescence measurements were done on a Shimadzu spectrophotometer model UV-260 (Shimadzu Corporation, Japan), Farrand system 3 (Farrand Optical Co., USA) spectrofluorimeter or Perkin-Elmer MPF 44A fluorimeter with phosphorescence attachment respectively. The details of the measurement procedure were described in (Maiti *et al* 1983, Chakraborty *et al* 1989b, Andley and Chakraborty 1982). Fluorescence quantum yields were calculated from the following equation as described (Maiti *et al* 1983) :

$$\theta_s = (F_s.\epsilon_q.C_q/F_q.\epsilon_s C_s) \times 0.55 \quad (1)$$

where s and q denote sample and quinine sulphate in 0.1 N H_2SO_4, F denotes the integral area of the fluorescence with excitation at the same wavelength, ϵ is at the wavelength of excitation and C represents the corresponding concentrations.

Fluorescence life time (τ_F) measurements were carried out at 22°C on a modified Ortec 9200 nanosecond fluorimeter by analysis the following equation as described by Tao and Cho (1979) :

$$1/\tau_F = 2.88 \times 10^{-9} n^2 \lambda^2 \int_0^{\alpha} \epsilon(\lambda)\, d\lambda \quad (2)$$

where n, ϵ and λ are the refractive index of the solvent, the molar extinction coefficient and the wavelength of maximum absorption respectively.

Phosphorescence life time (τ_P) measurements were calculated from the phosphorescence decay curves using a recorder at 240 mm/min from the following equation :

$$\tau_P = t/\ln(I_0/I) = 1/K \quad (3)$$

where I_0 and I are the intensity at time zero and at time t ; K is the decay constant.

Viability and morphological changes of the alkaloid treated EAC cells were examined under phase contrast with a Leitz Diaplan Scientific and clinical microscope, W. Germany with photographic attachment (Eaton *et al* 1959, Bekesi *et al* 1969, Hazra *et al* 1984). Suspension (ether in 0.145 M saline or RPMI medium) of the harvested cells were incubated in presence and absence of alkaloids (conc. 20 μg/10^6 cells/ml) for 1 hr at 37°C and were withdrawn at different time intervals. For viability test the viable cell (unstained by trypan blue) were counted by a haemocytometer. Viability was calculated as

$$\text{Viability } \% = (T/C) \times 100 \quad (4)$$

where T and C represent the surviving cells in a treated group and in untreated group respectively.

The effects of alkaloids on the transplantability of EAC cells was determined according to the procedure of Ritter *et al* (1987). After removal of extracellular alkaloids from the alkaloid treated cells the packed cells were suspended in 0.145 M NaCl solution and injected intraperitoneally into different groups (10 mice

in each group) of mice (10^6 cells/mouse). The mean life span (MLS) of each group was observed. Increase in life span (ILS) of the mice in different groups in comparison to the saline control was calculated using the formula :

$$ILS\ (\%) = [(T_t/T_0) - 1] \times 100 \quad (5)$$

where T_t and T_0 are the survival time of the treated and untreated group respectively.

The effects of alkaloids on the macromolecular synthesis of DNA, RNA and protein were assessed by measuring cellular incorporation of tritiated thymidine, uridine and leucine from culture medium. EAC cells (10^6 cells/ml) in presence or absence of alkaloids were incubated at 37°C for 1 hr in a shaking water bath and [^{3}H] thymidine or [^{3}H] uridine or [^{3}H] leucine (1 μCi/ml) were added. After 5 min intervals 1 ml of cell suspension was removed and diluted to 10 ml of PBS. Cells were then collected on Whatman GF/A glass microfibre filters and macromolecules were precipitated by the addition of cold 10% TCA. The residue were washed three times with 5 ml cold 5% TCA and rinsed with 5 ml 90% ethanol and dried overnight. The radioactivity of the dried filters were determined in a scintillation fluid (of 4 gm of PPO and 2 gm POPOP per litre toluene) using LKB Wallack 1217 RACK BETA liquid Scintillation counter. The inhibition of incorporation is expressed as the difference in incorporation between alkaloid treated and control cells.

3. Results

Characteristic data for absorbance, fluorescence and phosphorescence are presented in Table 1. The fluorescence quantum yield is maximum for AL and decreases

Table 1. Absorbance, fluorescence and phosphorescence parameters of alkaloids.

Parameters	AA	AL	ALM	ADG
Absorption maxima (nm) ($\epsilon \times M^{-1}\ cm^{-1}$)	398 (6456) 320 (13271) 266 (21062)	398 (6309) 330 (6841) 290 (12770) 259 (27421)	398 (6984) 330 (7368) 290 (13405) 259 (34025)	398 (10930) 330 (12004) 290 (19295) 259 (46954)
Fluorescence emission maximum (nm)	ND	485	485	485
ϕ_F ($\times 0^{-3}$)	ND	295	162	155
τ_F (ns)	ND	9.1	8.4	8.1
Phosphorescence emission maximum (nm)	—	575, 630	575, 630	575, 630
τ_P (s)	—	0.45	0.50	0.65

ND = Not detected.

in the order AL > ALM > ADG. It is interesting to note that the data of ϕ_F and τ_F decrease in the order AL > ALM > ADG while τ_P decreases in the reverse order.

Table 2. Effects of various alkaloids towards EAC cells.

Experiment with or without alkaloids	Trypan blue test viability	Loss of transplantability		Incorporation of radioactive precursors after 1 hr					
				[^{3}H] Thymidine		[^{3}H] Uridine		[^{3}H] Leucine	
		MLS (days)	ILS (%)	CPM/10^5 cells	Inhibition (%)	CPM/10^5 cells	Inhibition (%)	CPM/10^5 cells	Inhibition (%)
Control	99 ± 1	21 ± 1	0	6000 ± 150	0	15000 ± 1000	0	7000 ± 200	0
AA	97 ± 2	38 ± 1	81 ± 4	5000 ± 125	16.6 ± 2.0	14800 ± 600	2.0 ± 1.8	3900 ± 80	44.3 ± 0.7
AL	70 ± 3	26 ± 1	23 ± 1	3300 ± 90	44.4 ± 1.5	6400 ± 120	56 ± 3.2	5400 ± 150	22.8 ± 4.0
ALM	42 ± 5	24 ± 1	14 ± 1	3300 ± 100	44.4 ± 1.8	5000 ± 300	66.6 ± 0.7	5400 ± 150	22.8 ± 2.2
ADG	75 ± 2	37 ± 1	75 ± 2	2080 ± 60	65.27 ± 2.0	9200 ± 200	38.6 ± 2.0	5400 ± 200	22.8 ± 0.8

The data obtained from various biological experiments are presented in Table 2. It can be seen from the Table 2 that all alkaloids exhibits cytotoxic effect towards EAC cells. The viability percentage decreases in the order AA > ADG > AL > ALM while the transplantability is affected maximum for AA and then decreases in the order ADG > AL > ALM (Table 2). Morphological alterations of the EAC cells are observed in presence the alkaloids. After 1 hr treatment with AL, ADG and ALM (10 μg/10^6 cells) complete destruction of EAC cells is observed. In case of ADG, however, after 30 min treatment the EAC cells are found to be swollen. Very little morphological changes are observed in presence of AA.

Effect of the alkaloids on the macromolecular synthesis of EAC cells are studied and the summarized data are presented in Table 2. Time courses for synthesis of DNA is significantly affected with the alkaloids. The inhibition of DNA synthesis in the order ADG > AL > ALM > AA while RNA synthesis inhibition is in the order ALM > AL > ADG and it is unaffected with AA. The protein synthesis in the presence of the alkaloid is affected by a comparatively smaller degree except for AA where the protein synthesis is inhibited by about 44% τ_y and τ_p data presented here could not be correlated with their biological properties.

4. Discussion

There are several approaches that might be used to test the effect of chemotherapeutic drugs on cancer cells. Among them (i) measurement of morphological changes in tumour cells after drug exposure (Wright and Walker 1975, Balconi et *al* 1973), (ii) measurement of the increase in life span of test animals after implantation of drug treated tumour cells (Berry et *al* 1975, Salmon et *al* 1978, Ritter et *al* 1987), (ii) measurement of inhibition of cellular metabolism using radioactively labelled metabolic precursors (Philips 1974, Srivastava et *al* 1980) are often used. All these approaches have been utilized to test the antitumour effect of aristolochia group of alkaloids in the present study.

It can be seen from Table 2 that AA shows significant antitumour effect as revealed from transplantabiliiy experiment with almost minimal effects towards morphological change, loss of viability and inhibition of DNA synthesis. Similar observation has been reported by Kamatsh et *al* (1973) that growth of mouse sarcoma 37 cells incubated with AA was completely inhibited. It is interesting to note that AA does not form any complex with DNA or RNA (unpublished observation). So the cellular activity of AA may be due to some metabolite products of AA which significantly interfere in curing of infected mouse.

In the present study ADG, AL and ALM showed some inhibitory effect towards the growth of EAC cells both *in vivo* and *in vitro*. The cells affer ADG exposure showad swelling and subsequant disruption of cell structure ; while after AL and

ALM exposure, only disruption of cells had been observed. Cell survival as obtained from transplantability test is higher in ADG treated cells than with AL and ALM treated ones. Similar effect has been observed by Elaterician A and B (Gitter *et al* 1961) and various derivatives of Cucurbitacins (Gallily *et al* 1962). Although AA does not bind to DNA or RNA *in vitro*, it inhibits protein synthesis of EAC cells significantly while it has very little effect on DNA synthesis but no effect on RNA synthesis. The inhibition of thymidine and uridine incorporation suggest that all these lactans act in cellular level and binds to nucleic acid. Incorporation experiments with EAC cells indicate that AL, ADG and ALM disrupt DNA and RNA synthesis with less effect on protein synthesis. This is comparable to the effect of ellipticine (Li and Cowie 1974). Interestingly, ADG show lower inhibitory effect on RNA synthesis than that on DNA synthesis in contrast to the intercalator adriamycin which inhibit DNA and RNA synthesis equally in intact cells. However, certain intercalating anthracyclines show more inhibition in DNA synthesis (Crooke *et al* 1981). Dercitin, a new acridine alkaloid isolated from a marine Dercitus species sponge shows greater inhibition of RNA synthesis (Burres *et al* 1989) and it is comparable to the data presented for AL. The binding of ADG to DNA has been reported (Chakraborty *et al* 1989a, 1990) and similar binding of AL, ALM has been observed (unpublished). Thus, the incorporation studies are in agreement with the intracellular binding of AL, ALM, ADG to nucleic acid and explain the disruption of the integrity of the cell.

Acknowledgments

S Chakraborty and R Nandi are grateful to the Council of Scientific and Industrial Research, Government of India for the award of Senior Research Fellowship and Research Associateship respectively. The authors express their gratitude to Dr B Chakraborty, Boston, USA for his help to measure fluorescence life time and phosphorescence spectra.

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